Biomaterials xxx (2014) 1–8

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of keratinocytes on the biomechanical characteristics and pore microstructure of tissue engineered skin using deep dermal fibroblasts

Mathew Varkey [a], Jie Ding [a], Edward E. Tredget [a, b, *], Wound Healing Research Group

[a] Division of Plastic and Reconstructive Surgery, University of Alberta, 2D3.81 WMSHC, 8440-112 Street, Edmonton, Alberta T6G 2B7, Canada
[b] Critical Care Medicine, University of Alberta, 2D3.81 WMSHC, 8440-112 Street, Edmonton, Alberta T6G 2B7, Canada

ARTICLE INFO

Article history:
Received 11 June 2014
Accepted 23 July 2014
Available online xxx

Keywords:
Tissue engineered skin
Biomechanical characteristics
Pore microstructure
Keratinocytes

ABSTRACT

Fibrosis affects most organs, it results in replacement of normal parenchymal tissue with collagen-rich extracellular matrix, which compromises tissue architecture and ultimately causes loss of function of the affected organ. Biochemical pathways that contribute to fibrosis have been extensively studied, but the role of biomechanical signaling in fibrosis is not clearly understood. In this study, we assessed the effect keratinocytes have on the biomechanical characteristics and pore microstructure of tissue engineered skin made with superficial or deep dermal fibroblasts in order to determine any biomaterial-mediated anti-fibrotic influences on tissue engineered skin. Tissue engineered skin with deep dermal fibroblasts and keratinocytes were found to be less stiff and contracted and had reduced number of myofibroblasts and lower expression of matrix crosslinking factors compared to matrices with deep fibroblasts alone. However, there were no such differences between tissue engineered skin with superficial fibroblasts and keratinocytes and matrices with superficial fibroblasts alone. Also, tissue engineered skin with deep fibroblasts and keratinocytes had smaller pores compared to those with superficial fibroblasts and keratinocytes; pore size of tissue engineered skin with deep fibroblasts and keratinocytes were not different from those matrices with deep fibroblasts alone. A better understanding of biomechanical characteristics and pore microstructure of tissue engineered skin may prove beneficial in promoting normal wound healing over pathologic healing.

1. Introduction

Wound healing in humans occurs generally through fibrosis and less often through regeneration. Fibrotic conditions affect most organs and results in the progressive replacement of normal parenchymal tissue with collagen-rich extracellular matrix that is characteristic of connective tissue [1]. In its most severe clinical manifestations, fibrosis may either cause cosmetic and functional problems as seen in skin fibroproliferative disorders [hypertrophic scars (HTS) and keloids] or organ failure as in idiopathic pulmonary fibrosis, liver cirrhosis, cardiovascular fibrosis, renal fibrosis, and scleroderma. Chronic fibrotic diseases are believed to be the leading cause of morbidity and mortality worldwide with current estimates suggesting a role in 45% of all deaths in the developed world [2]; however, unfortunately very limited effective therapies are currently available for treatment of fibrotic diseases.

In normal tissue, a fine balance between synthesis and degradation of collagen, the main component of extracellular matrix (ECM), helps maintain physiological homeostasis. However during wound healing, the equilibrium is shifted towards accelerated collagen synthesis to aid tissue repair. In the case of fibrosis, collagen homeostasis is not restored at the culmination of the wound healing process, which results in excessive accumulation of collagen, hypercellular regions of fibroblasts and disorganized ECM [3]. Fibrosis could lead to loss of function of the associated organ, and can be either local as in the case of the skin fibroproliferative disorders, hypertrophic scars (HTS) and keloids or systemic as in scleroderma [4–6]. Liver fibrosis interferes with drug metabolism causing accumulation of toxic metabolites, and lung fibrosis causes poor blood-gas exchange resulting in hypercarbia and/or hypoxia [7,8]. HTS results in functional limitations of range of motion as well as cosmetic problems like color mismatch, stiffness, and rough texture, in addition to itching and pain [9]. In all, fibrosis has a

* Corresponding author. University of Alberta, 2D3.81 WMSHC, 8440-112 Street, Edmonton, Alberta T6G 2B7, Canada. Tel.: +1 780 407 6979; fax: +1 780 407 7394.
E-mail address: etredget@ualberta.ca (E.E. Tredget).

http://dx.doi.org/10.1016/j.biomaterials.2014.07.048
0142-9612/

significant impact on the outcome of wound healing, severely affects the quality of life of affected patients and leads to significantly increased health-care costs.

Fibrosis occurs as an aftermath of inflammatory and connective tissue repair response mechanisms that facilitate physiological repair of the body. It can be triggered by a variety of stimuli including persistent infections, autoimmune reactions, chemical insults, and tissue injury due to burns or other causes [4]. The key cellular mediator of fibrosis, myofibroblast, exhibits characteristics of both fibroblasts and smooth muscle cells. Myofibroblasts can be derived from resident mesenchymal cells, epithelial and endothelial cells, or fibrocytes [4]. They can be activated by a variety of mechanisms, including autocrine factors, paracrine signals derived from lymphocytes and macrophages, and molecular patterns produced by pathogens. They are highly contractile in nature, and generate connective tissue contracture and irreversible ECM remodeling producing stiff fibrotic scars. There is accumulating evidence that biomechanical, geometrical and topographical cues from the local environment are important for modulating cell behavior and growth. Matrix topography affects a whole range of cell responses including cell attachment, migration, differentiation and new ECM production [10,11]. The ability of the tissue to regenerate itself or undergo abnormal repair may be dependent on the local microenvironment provided by the scaffolds during the initial phase of the injury. Currently, there is limited information about how biomechanical and topographical cues promote normal wound healing over abnormal wound repair.

Also, there is very little information about the combined influence of scaffold microtopography and biomechanical characteristics on wound healing. An effective mode of promoting regenerative wound healing over fibrotic healing may be by manipulating the inherent physical properties of the biomaterial scaffold which would present an easily translatable therapeutic innovation. In this study we assessed the effect of two different cell populations on substrate properties. This system affords the ability to study the physiological scenario in a native-like microenvironment. Biophysical signaling is believed to regulate cell behavior and characteristics either through external mechanical forces or through intrinsic matrix stiffness [12].

The biomechanical properties of the native ECM are understood to be tightly controlled not only by specific composition of matrix constituents but also by post-translational modifications, cross-linking, transglutamination and glycosylation [13]. In this study, we assessed the effect keratinocytes have on the biomechanical characteristics and pore microstructure of tissue engineered skin with superficial or deep dermal fibroblasts to determine if there were any biomaterial-mediated anti-fibrotic influences on tissue engineered skin.

2. Materials and methods

2.1. Preparation of C-GAG matrices

Acellular C-GAG matrices were prepared by freeze-drying a co-precipitate of type I collagen and chondroitin-6-sulfate as reported previously [14]. Briefly, collagen powder (0.5 wt %; Devro Pty. Ltd., Bathurst, NSW, Australia) was co-precipitated with chondroitin-6-sulfate (0.05 wt %; Sigma, St. Louis, MO, USA) in 0.5 M acetic acid, degassed under vacuum (2 h, room temperature), cast into sheets, frozen to −40 °C and freeze-dried (FreeZone^{6}Plus, Labconco, Kansas City, MI, USA) to produce highly porous matrices. The matrices were cut into 30 mm discs and cross-linked by dehydrothermal treatment in a drying oven (vacuum, 140 °C, 48 h; APT.Line VD, Binder GmbH, Germany). Subsequently, the matrix discs were rinsed with phosphate buffered saline (PBS; 2 times for 15 min each) and cell culture medium (DMEM, 10% FBS, 1% antibiotic-antimycotic; 2 times for 15 min each).

2.2. Preparation of different types of tissue engineered skin

Tissue engineered skin was prepared by co-culturing superficial or deep dermal fibroblasts with keratinocytes on dehydrothermal-treated C-GAG matrices. The cells were obtained from lower abdominal tissue of three patients who underwent elective abdominoplasty surgery following informed consent. The protocols for human tissue sampling used in this study were approved by the University of Alberta Hospital's Health Research Ethics Board. Superficial and deep dermal fibroblasts and keratinocytes were isolated as reported previously [14]. Briefly, tissue samples were horizontally sectioned into five layers (referred to as layers 1–5) using a dermatome (Padgett Instruments, Plainsboro, NJ, USA) set approximately at 0.5 mm. The superficial dermal layer (layer 1) was treated overnight with dispase (25 U/mL, 4 °C; Gibco, Grand Island, NY, USA) to remove the epidermis, which was digested with trypsin to isolate keratinocytes. On the other hand, the superficial dermal layer and the deep dermal layer (layer 5) were separately treated with collagenase (455.3 U/mL, 18 h, 37 °C, 60 rpm; Gibco Grand Island, NY, USA) to isolate the superficial (SF) and deep (DF) dermal fibroblasts, respectively. The keratinocyte (K) and fibroblast cell suspensions were passed through 100 μm cell strainers and centrifuged at 800 rpm for 10 min. The epidermal and dermal cell pellets were then re-suspended in the respective cell culture media and serially expanded in tissue culture flasks until the desired number of K, SF and DF were obtained. Passage 4 SF or DF were seeded onto cross-linked C-GAG discs at a density of 0.5×10^6 cells/cm^2 and cultured at 37 °C, in 5% CO_2. Three days later, K were seeded on top of the fibroblast-populated matrices at a density of 1.0×10^6 cells/cm^2 and medium was replaced with co-culture medium containing serum (DMEM-HG and nutrient mixture F-12 Ham (3:1), 2 nM triiodothyronine, 5% FBS, 0.5% insulin–transferrin–selenium-G supplement, 1 nM cholera toxin, 10 ng/ml EGF, 0.4 μg/ml hydrocortisone, 5 μg/ml transferrin, 1% antibiotic-antimycotic [15]). Tissue engineered skin made of SF, DF and K was used as a control for tissue engineered skin made of heterogeneous fibroblasts while matrices with SF or DF alone were used as K-free controls, where the number of cells was adjusted so as to make the total number of cells on the matrices comparable to those that had K. The submerged culture was continued for 5 additional days and then lifted to the air-liquid interface on a steel platform to enable epidermal stratification. The tissue engineered skin and controls were used in assays at different time points as described below.

2.3. Immunofluorescent staining

5 mm punch biopsies were collected from the different tissue engineered skin constructs and controls at days 7, 14 and 21 of culture for immunohistochemical staining of α-smooth muscle actin (SMA) or cytokeratin. α-SMA and cytokeratin, are specific markers for myofibroblasts and keratinocytes, respectively. Briefly, the samples were fixed with 4% paraformaldehyde (12 h) and 70% ethanol (12 h), paraffin embedded and sectioned at 5 μm and mounted on microscope slides. The sections were then deparaffinized with xylene and rehydrated in descending series of ethanol, and subsequently blocked with 10% BSA in PBS for 60 min to avoid non-specific protein binding. The sections were incubated overnight with primary mouse anti-α-SMA or anti-cytokeratin antibody (4 °C, 1:50 dilution; Dako, Denmark), and later washed with PBS (3 times for 5 min). Non-immune goat IgG at 1:50 dilution was used as the negative control. Endogenous peroxide activity was quenched with 0.3% H_2O_2 (15 min) and subsequently the sections were incubated with fluorescent secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG (60 min, room temp., 1:500 dilution; Invitrogen, Oakville, ON, Canada). The stained sections were then mounted with DAPI containing ProLong Gold reagent, and viewed by fluorescent microscopy at 100 × magnification and photographed.

2.4. Analysis of contraction of tissue engineered skin

To determine the extent of contraction, the different tissue engineered skin were photographed on days 7, 14 and 21, and the images were analyzed using NIH image J. The values are reported as mean percentage contraction ± standard error.

2.5. Biomechanical testing of the different tissue engineered skin

The biomechanical properties of the tissue engineered skin constructs were determined by subjecting them to tensile testing. Briefly, C-GAG matrices were cut into dog-bone shaped pieces (gauge length of 50 mm and width of 15 mm) and were cross-linked. Subsequently, superficial and deep dermal fibroblasts were independently co-cultured with or without keratinocytes on the cross-linked matrices. On days 7 and 21 of culture, the matrices were mounted onto the grips of an Instron tensile tester (Instron, Norwood, MA, USA) connected to a 50 N load cell and were tested at a strain rate of 2 mm/min. Tensile strength assessment of the matrices were done on days 7 and 21 in order to assess earlier and later cellular effects on biomechanical characteristics of the matrices. The tensile tests were set up such that all the samples were strained to failure. C-GAG matrices without cells were used as cell-free controls. The ultimate tensile strength (UTS; kPa) and stiffness (mN/mm) values were calculated and are reported as mean ± standard error.

2.6. Gene expression studies

5 mm punch biopsies were collected from the different tissue engineered skin and the controls at days 7, 14 and 21 of culture and snap frozen in liquid nitrogen for subsequent gene expression analysis. The frozen matrices were homogenized (2000 rpm, 2 min; Mikro-Dismembrator S, B. Braun Biotech International), and treated with Trizol reagent (Invitrogen, Carlsbad, CA, USA); the resultant supernatant was stored at −80 °C for subsequent analyses. Later, total RNA was extracted

Please cite this article in press as: Varkey M, et al., The effect of keratinocytes on the biomechanical characteristics and pore microstructure of tissue engineered skin using deep dermal fibroblasts, Biomaterials (2014), http://dx.doi.org/10.1016/j.biomaterials.2014.07.048